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Research article

Angiotensin I-converting enzyme (ACE) inhibitory activities of enzymatically hydrolyzed jackfruit seed protein

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Abstract

Importance of the work: Angiotensin-converting enzyme (ACE) has been well-recognized for its role in blood pressure regulation. Jackfruit seed protein hydrolysates (JSPs) are produced via enzymes.

Objectives: To assess the potential of jackfruit seed proteins as a functional food for high blood pressure treatment based on bioactive peptides.

Materials and Methods: The ACE inhibitory activities were investigated of the JSPs produced via pepsin, trypsin and chymotrypsin enzymes. After hydrolysis, smaller JSP molecules (≤ 3 kDa) were collected following ultrafiltration and further purified using solid phase extraction (SPE). The ACE inhibitory activity analysis of all peptides revealed positive results. Hence, the peptides were further separated into fractions using hydrophilic interaction liquid chromatography (HILIC) and reversed phase-high performance liquid chromatography (RP-HPLC) and subsequently, analysis of the inhibitory properties of JSP fractions against ACE was carried out.

Results: The mean (± SD) yield of protein from jackfruit seeds extracted using sonication at 30% amplitude was 18.52 ± 0.6% dry basis. The JSPs had a mean (± SD) ACE-inhibitory activity of 77.75 ± 0.17% and a half maximal inhibitory concentration (IC_{50}) of 0.54 mg/mL. The impediment to ACE activity by the HILIC 90%_JSP_ACN+0.1%FA fraction was the highest. Based on further RP-HPLC fractionation of this HILIC fraction, the inhibition efficiency of fraction 6 (HILIC90%_JSP_RP-HPLC_6) against ACE was exemplary.

Main finding: JSPs are promising alternative sources of antihypertensive functional food, food ingredients and nutraceuticals.

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Introduction

Noncommunicable diseases (NCDs) are diseases that arise mainly due to unhealthy behaviors and sometimes genetic and environmental issues or a combination of these three factors (Calcaterra and Zuccotti, 2022). NCDs, mainly cardiovascular diseases, cancers, diabetes and respiratory diseases, are collectively responsible for over 70% of all deaths worldwide (World Health Organization, 2023). Among these, hypertension, commonly known as high blood pressure, is a serious chronic disease that usually develops over time, arising when the force of blood pushing against artery walls is constantly too elevated (World Health Organization, 2023). According to the World Health Organization, hypertension is pervasive among people aged 30–80 yr in low- and middle-income countries (World Health Organization, 2023). Likewise, there has been a rising prevalence of hypertension among the Southeast Asian population, including Thailand (Aekplakorn, 2019). The World Health Organization (2023) predicted that 1.56 billion people would be diagnosed with hypertension by 2025, while in Thailand, the number of people with hypertension was predicted to increase from 3,936,171 to 5,597,671 between 2013 and 2017 Furthermore, the prevalence of hypertension among Thai adults was projected to increase from 21.41% to 24.71% between 2009 and 2014 (Strategy and Planning, Ministry of Public Health, 2015). These alarming statistical predictions have triggered ample research to uncover natural bioactive compounds for preventing and decreasing blood pressure.

Jackfruit (*Artocarpus heterophyllus* Lam.), the largest-sized fruit in Thailand, is a major fruit crop that is processed and canned for export markets (Posomboon, 2015), with the related processing industries generating a considerable amount of unused peels, cores and seeds that could be reutilized as an ingredient in different sustainable products (Wongarun, 2019). Jackfruit consists of 10–15% seeds which are rich in starch (22%), protein (17.8%), fiber (3.19%), phosphorus and vitamin B1 (Department of Agriculture, 2008; Swami et al., 2012; Ulloa et al., 2017; Wongarun, 2019). The naturally high protein content of jackfruit seeds makes them suitable for proteolysis. The use of protein hydrolyses dates back to the 1940s when it was used as a nutritional supplement for protein-intolerant individuals (Cuthbertson, 1950). Since then, research on these hydrolyses has led to their introduction into the healthcare, cosmetics and food industries (Radhe et al., 2007). Bioactive peptides are a group of organic molecules, within the parent protein that are composed of amino acids bonded covalently by amide or peptide bonds that when cleaved using hydrolysis, become active and beneficial to the human body and health. Bioactive peptides can prevent oxidation and microbial degradation in foods and can be used to treat various medical conditions, thus increasing the quality of human life (Lemes et al., 2016). Bioactive peptides are well known for their use in the prevention of illnesses such as hypertension, diabetes, Alzheimer's disease and cancer (Udenigwa and Aluko, 2012). Hypertension may be influenced by several health factors, including the renin-angiotensin-aldosterone system (RAAS) which is responsible for the basic regulation of blood pressure through the control of fluid volumes in the body. Angiotensin-converting enzyme (ACE) is known for regulating a balance between the functions of angiotensin II (vasoconstriction and salt-retentive properties) and bradykinin (vasodilators and natriuretic properties) (Hollengerg, 2000; Maja and Josef, 2019). Extensive studies have been carried out to identify ACE inhibitors (ACEI) from natural sources to replace synthetic ones such as captopril, enalapril and lisinopril. For example, recently, natural-sourced bioactive peptides have been examined extensively because of their potential ACE inhibition properties. The present study was the first known of its kind to evaluate the potential of bioactive peptides from jackfruit seed proteins as ACE inhibitors.

Sample preparation for seed protein extraction is also regarded as a purification process before instrumental analysis. It involves separating and enriching targets from a complex sample while eradicating or lessening interference through the use of solvent and adsorbent-based sample pretreatment techniques to be compatible with instrument analysis (Tang et al., 2017; Che et al., 2017). Some of the combined techniques and analytical instruments applied in seed protein hydrolysate analysis include liquid-liquid extraction, liquid-liquid micro-extraction, solid-phase extraction (SPE), strong cation exchange, hydrophilic interaction liquid chromatography (HILIC) and reverse phase-high performance liquid chromatography (RP-HPLC) (Kleekayai et al., 2014; Ngamsuk et al., 2020; Chai et al., 2021).

The present study used a combination of SPE, HILIC and RP-HPLC to purify jackfruit seed protein hydrolysates. Bioactive characterization of the purified fractions was evaluated based on their ACE inhibitory activity.

Materials and Methods

Materials

The jackfruit seeds (Thong Prasert jackfruit) were collected from Prachuap Khiri Khan Province, Thailand. Acetone, acetonitrile (ACN), methyl alcohol (MeOH) and trichloroacetic acid (TCA) were purchased from RCI Labscan (Bangkok, Thailand). Hexane was purchased from Fisher Scientific (Seoul, Republic of Korea). Formic acid (FA), sodium chloride (NaCl), sodium hydroxide (NaOH), boric acid, potassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were purchased from KemAus (West Pennant Hills, NSW, Australia). Pepsin, trypsin, chymotrypsin, hippuryl-L-hidtidyl-lleucin (HHL), angiotensin-converting enzyme and captopril were purchased from Sigma-Aldrich (St Louis, MO, USA). Sodium hydroxide and citric acid (food grade) were purchased from AGC Chemicals (Pathumwan, Bangkok, Thailand). Hydrochloric acid (HCl) was purchased from QREC Chemicals (New Zealand). Ferulic acid and solid-phase extraction (SPE) columns were purchased from Acros, Thermo Scientific (TN, USA). Ethyl alcohol was purchased from Krungthepchemi(Bangkok, Thailand). Trifluoroacetic acid (TFA) was purchased from Fisher Scientific (Loughborough, UK). Ultra-filtration membranes (3 kDa MWCO) were purchased from Millipore (Bedford, MA, USA). Hydrophilic interaction liquid chromatography (HILIC) and VertiSep™ BIO C18 HPLC columns were purchased from Vertical Chromatography (Nonthaburi, Thailand) and the C18 column Zorbax Eclipse 5 XDB-C18 (particle size 5 µm; 4.6 mm × 250 mm) was purchased from Agilent Technologies (city, CA, USA). Any other chemicals used were of analytical grade. Deionized water was utilized throughout the experiment.

Sample preparation and protein extraction

Fresh jackfruit seeds (1 kg) were washed thoroughly with water (4 L) before removing the white seed coating. Then, samples were processed for removal of the brown seed coat by soaking in 5% NaOH (4 L) for 2 min followed by 2 min in 5% citric acid (4 L) and then washed with water (4 L). The clean jackfruit seeds were sliced and tray-dried (Binder FE400; Scientific Promotion Co., Ltd; Bangkok, Thailand) at 45°C for 16 hr to a moisture content of less than or equivalent to 13% (adopted with slight modification from Rengsutthi and Charoenrein, 2011 and Akter et al., 2020). The dried jackfruit seeds were ground using a laboratory grinder (Powder grinder ECO-1500; Spring Green; Bangkok, Thailand) and then defatted according to the method of Castillo et al. (2017) in which the jackfruit seed powder-to-hexane ratio was 1:2 (weight per volume) at room temperature for 1 hr. Subsequently, the defatted sample was dried in a fume hood (Easy Lab FH509; S.K. Powerable Co., Ltd; Samut Sakhon, Thailand) for 24 hr. The defatted jackfruit seed powder (DJSP) was kept in a desiccator until further use. Proteins were extracted using ultrasonication based on the method of Ngamsuk et al. (2020). Proteins from the 6 mg of the DJSP were extracted using a 20 kHz sonicator (Branson Digital Sonifer®; Emerson Electric Co.; St. Louis, MO, USA) at 30 % amplitude and pulse on/off at 20/10 s (3 min) in deionized water (6 mL) at 0°C. The solutions were centrifuged (Kubota High Speed Refrigerated Centrifuge, model 6000; Bara Scientific & Co. Ltd; Bangkok, Thailand) at 4°C and 15,000×*g* for 10 min. The resultant supernatants were collected, freeze-dried (Heto LyoLab 3000 Freeze dryer; Scientific Promotion Co. Ltd; Bangkok, Thailand) and stored at -20°C. The proteins were precipitated with 10% TCA in acetone at a 1:1 (volume per volume) ratio at -20°C for 24 hr, centrifuged at 4°C and 15,000×*g* for 10 min and washed three times with acetone (3 mL). Subsequently, the protein pellet was washed with deionized water, centrifuged again at 4°C and 15,000×*g* for 10 min and freeze-dried and stored at -20°C. Hereafter, this powder was referred to as the jackfruit seed protein (JSP) extract in subsequent experiments.

Jackfruit seed protein hydrolysate

The JSP was hydrolyzed using a combination of enzymes (pepsin, trypsin and chymotrypsin). The JSP powder (10 mg) was mixed with *35* mM NaCl and adjusted to pH *2*. Pepsin (*250* units/mg) was added to the mixture and incubated in a shaker at 200 revolutions per minute (rpm), *37*°C for *24* hr (Innova 4230 New Brunswick Scientific Refrigerated Incubator Shaker; Scientific Promotion Co., Ltd; Bangkok, Thailand). Then, the pepsin was inactivated by adjusting the mixture to pH *8.0*. Trypsin and chymotrypsin (*250* units/mg, respectively) were added to the JSP mixture and incubated at *37*°C for *24* hr. Hydrolysis was terminated by heating the JSP mixture at *95*°C for *15* min and cooling at *5*°C for *10* min, followed by filtration (ultra-filtration membrane ≤ *3* kDa; Millipore, MA, USA) and centrifugation using the Kubota High Speed Refrigerated Centrifuge described above at *10,000*×*g* for *10* min. The JSPHs in triplicates were lyophilized and kept at *-20*°C for further experiments.

Purification of jackfruit seed protein hydrolysates using solid-phase extraction

The JSPHs were dissolved in deionized water and desalted using an SPE column (HyperSep™ C18; Thermo Scientific; TN, USA), according to Ngamsuk et al. (2020). The SPE columns were conditioned with MeOH and ACN + 0.1%FA and then equilibrated with 5%ACN + 0.1%FA. The JSPHs were loaded to the preconditioned SPE column and flushed with three column volumes of 0.5%ACN + 0.1% FA. Subsequently, the column was flushed with 50% ACN+0.1%FA and the eluted peptides were collected. Then, the column was flushed with 80%ACN+0.1%FA to obtain another fraction. The eluted peptides were combined in triplicates, freeze-dried and kept at -20°C for further experiments.

Purification of eluted peptides using hydrophilic interaction liquid chromatography

The eluted peptides were dissolved with 95%ACN+0.1%FA and purified using HILIC (Vertical Chromatography; Nonthaburi, Thailand), according to Ngamsuk et al. (2020). The HILIC columns were activated by flushing with MeOH and 5%ACN+0.1%FA and then equilibrated with 95%ACN+0.1%FA. The peptides were sequentially eluted from the pre-activated HILIC column with 100%ACN+0.1%FA, 90%ACN+0.1%FA, 80%ACN+0.1%FA, 70%ACN+0.1%FA, 60%ACN+0.1%FA, 50%ACN+0.1%FA and 0%ACN+0.1%FA (100%deionized water+0.1%FA; no ACN), freeze-dried and kept at -20°C. Each eluted fraction was collected in triplicates for further experiments.

Fractionation of jackfruit seed peptides

Preliminary studies indicated that the HILIC 90%ACN+0.1%FA fraction had the highest ACE inhibitory activities. Therefore, this fraction was further separated using RP-HPLC. The HILIC 90%ACN+0.1%FA sample was dissolved with 5%ACN+0.1%FA, injected and separated using HPLC (Agilent 1260 Infinity HPLC; Agilent Technologies; CA, USA) with a C18 column described above, according to Ngamsuk et al. (2020). The mobile phase consisted of 5%ACN+0.1%TFA (solvent A) and 95% ACN+0.1%TFA (solvent B). The flow rate was set at 1 mL/min and the peptides were separated according to the following gradient: 100% solvent A from 0 min; 100–80% solvent A from 0–45 min; 80–20% solvent A from 45–85 min and 20% solvent A from 85–90 min. The fractions in triplicates were monitored at a wavelength of 214 nm. The peptide fractions were collected every 5 min, freeze-dried and kept at -20°C for further analysis.

Angiotensin I-converting enzyme inhibitory activity analysis of purified fractions

Each peptide sample was dissolved in deionized water and added to 2.5 mM hippuryl-L-histidyl-L-leucine. All samples were pre-incubated at 37°C for 5 min before adding 0.05 mU/µL of ACE and subsequently incubated at 37°C for 30 min in a shaker incubator (Innova 4230 New Brunswick Scientific Refrigerated Incubator Shaker; Scientific Promotion Co., Ltd; Bangkok, Thailand) at 200 rpm. The reaction was halted with 1 N HCl; ferulic acid (0.25 mg/mL) served as the internal standard. All samples were centrifuged at 15,000 rpm for 2 min and the ACE inhibitory activity was examined in triplicate using RP-HPLC with the Agilent 1260 Infinity equipment described above fitted with the C18 column described above, using an isocratic gradient of 95% solvent A (5%ACN + 0.1%TFA) and 15% solvent B (95%ACN+0.1%TFA) at a flow rate of 1 mL/min. The samples were measured using an emission wavelength of 228 nm. and recorded. Borate buffer was used as the blank and captopril was used as the positive control.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. The SPSS software (version 17; SPSS Inc.; Chicago, IL, USA) was used for all statistical analyses. Differences among means were considered significant at $p < 0.05$. Results were presented as mean ± SD.

Results and Discussion

Protein extraction from jackfruit seeds

The yield of proteins from the jackfruit seeds extracted using sonication at 30% amplitude was 18.52 ± 0.06% on a dry basis. Similarly, Kumar et al. (1988) reported that the yield of jackfruit seed proteins was 17.8–18.3% of total compounds, while Miah et al. (2017) reported jackfruit seeds contained 16.01 ± 0.11% protein. These comparable protein extraction results indicated that jackfruit seeds were suitable for use in further experiments.

Angiotensin I-converting enzyme inhibitory activities of jackfruit seed protein hydrolysates

Quirós et al. (2007) reported higher ACE inhibitory activities for low molecular weight peptides than for higher peptides. Hence, the JSPHs in the present study were hydrolyzed based on the combination of the three enzymes followed by ultrafiltration. The JSPHs showed ACE-inhibitory activities of 77.75 ± 0.17% (Fig. 1) compared with captotril (93.82 ± 0.36%). The IC_{50} of the JSPHs was determined using different concentrations to give a value of 0.54 mg/mL. Similar results by Ambigaipalan et al. (2015) showed that date seed protein hydrolysate from combined enzymes (alcalase and thermolysin) had higher ACE inhibition activity (IC_{50} of 0.53 mg/mL) than single enzymes (alcalase: IC_{50} of 0.80 mg/mL; flavourzyme: IC_{50} of 1.57 mg/mL; and thermolysin: IC_{50} of 2.08 mg/mL). Furthermore, Lu et al. (2021) showed the sesame protein hydrolysate from dual enzymes (alcalase and trypsin) had higher ACE inhibition activity (83.22 ± 0.79-88.38 ± 0.65%) than single enzymes (alcalase, 45%; and trypsin, 50%). Wang et al. (2020b) reported that simulated digestion with trypsin and chymotrypsin significantly improved the ACE-inhibitory activity of sesame protein hydrolysates, indicating that enough short-chain peptides from JSPHs could be obtained through gastrointestinal protease digestion. According to other research, the antihypertensive activity from peptides could be related to the size, specific amino acid configuration, sequence length and hydrophobic or hydrophilic residues (Wijesekara and Kim, 2010; Udenigwe and Mohan, 2014; Wang et al., 2020a).

Angiotensin I-converting enzyme inhibitory activity of hydrophilic interaction liquid chromatography-purified jackfruit seed protein hydrolysate

HILIC is suitable for the separation of polar and hydrophilic compounds. Eight fractions were obtained from the JSPHs using the HILIC column and were studied using ACE inhibitory assay. The HILIC_JSPHs_90%ACN+0.1%FA had the highest ACE inhibitory activity (89.45 ± 0.36%) but was less than for captotril (97.96 ± 0.06%), with an estimated IC_{50} value of 0.074 ± 0.56 mg/mL (Fig. 2). The 90%ACN+0.1%FA HILIC fraction contained predominantly organic solvent, suggesting the possibility of both hydrophobic and hydrophilic peptides.

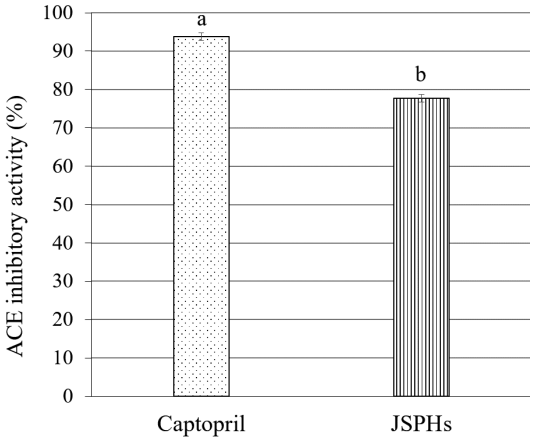

Fig. 1 Angiotensin I-converting enzyme inhibitory activity of jackfruit seed protein hydrolysates (JSPHs) compared to captopril.

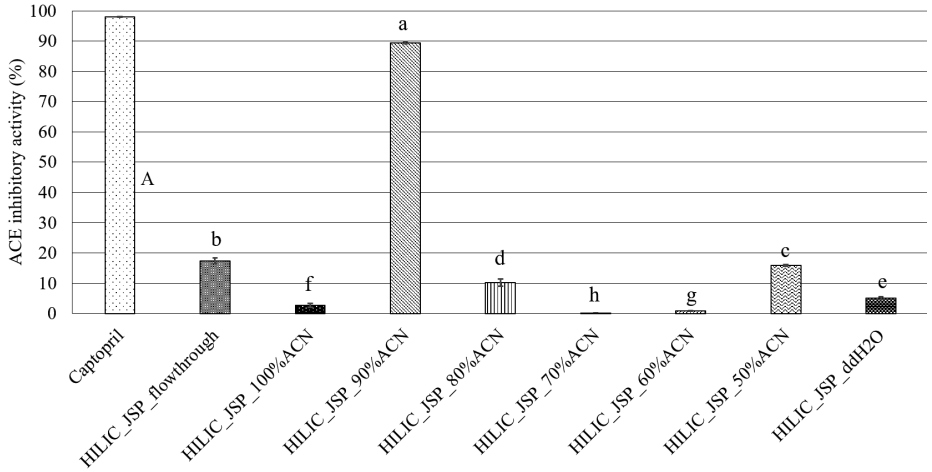

Fig. 2 Angiotensin I-converting enzyme (ACE) inhibitory activities of different hydrophilic interaction liquid chromatography (HILIC) fractions derived from jackfruit seed protein hydrolysates (JSPHs) < 3 kDa, where different lowercase letters above columns indicate significant ($p < 0.05$) differences. Error bars = ± SD.

In addition, the available evidence suggested that typically, peptide sequences exhibiting *in vitro* ACE inhibitory activity contain specific amino acids such as hydrophobic or aromatic residues (Zheng et al., 2022). This was also supported by the increased retention time in an HILIC study with hydrophilic peptides (Cubbon et al., 2007). Therefore, overall, it could be postulated that the peptides in the HILIC_JSPHs_90%ACN+0.1%FA fraction likely had specific hydrophilic and hydrophobic peptides that contributed to their strong ACE inhibitory activity. Therefore, the HILIC_JSPHs_90%ACN+0.1%FA fraction was selected for further experiments.

Angiotensin I-converting enzyme inhibitory activities of HILIC_JSPHs_90%ACN+0.1%FA fractions derived using reversed phase-high performance liquid chromatography

The HILIC_JSPHs_90%ACN+0.1%FA sample had the highest ACE inhibitory activity and therefore the highest potential to protect against hypertension. The HILIC_JSPHs_90%ACN+0.1%FA sample was further fractionated using RP-HPLC to produce 18 fractions (Fig. 3), where each fraction is indicative of different hydrophobicity and structural complexities. The percentages of ACE inhibitory activity for these fractions were: 60.92 ± 0.47, 2.67 ± 0.87, 36.63 ± 0.97, 56.46 ± 1.64, 29.78 ± 1.37, 64.43 ± 0.73, 59.33 ± 0.53, 41.00 ± 0.16, 41.75 ± 0.85, 42.02 ± 1.13, 30.37 ± 0.62, 29.58 ± 1.50, 29.68 ± 0.71, 22.95 ± 0.56, 35.92 ± 0.64, 30.21 ± 0.66, 26.16 ± 0.26 and -200.55±2.56%, while the activity for captopril was 90.89 ± 0.64%. The highest ACE inhibitory activity was expressed by fraction 6 (HILIC90%_JSPHs_RP-HPLC_6). Peptides derived from the combination with enzyme hydrolysis had high ACE inhibitory activities, possibly due to the presence of typical hydrophobic amino acids in the ACE inhibitor peptides, underpinning this fractions potential therapeutic applications, particularly in the development of natural antihypertensive agents. (Jung et al., 2006; Saito, 2008; Erdmann et al., 2008; Hanafi et al., 2018).

Conclusion

The peptides (< 3 kDa) hydrolyzed from jackfruit seed proteins had strong ACE inhibitory properties (IC_{50} = 0.54 mg/mL). These peptides were further separated using HILIC, with these results indicative of high inhibition against ACE by one of the fractions (HILIC_JSPHs_90%ACN+0.1%FA). Subsequent fractionation using RP-HPLC revealed that the HILIC_JSPHs_90%_RP-HPLC_6 fraction was the most potent ACE inhibitor. This finding indicated that the peptides isolated from jackfruit seed protein have exceptional potential for antihypertension and may be a suitable resource of functional peptides for drug applications.

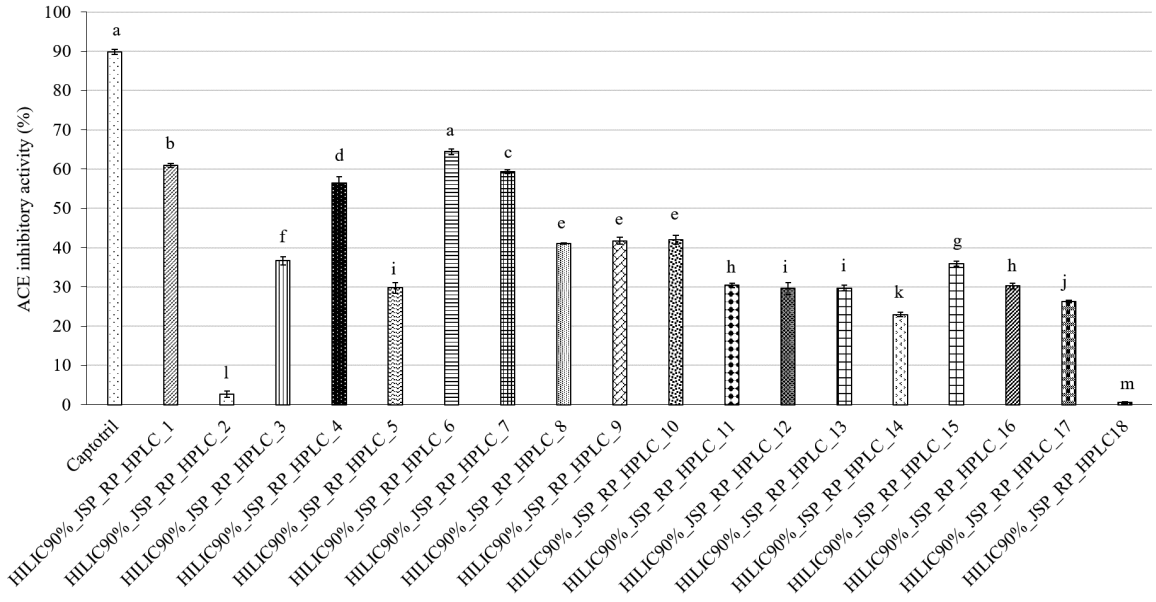

Fig. 3 Angiotensin I-converting enzyme (ACE) inhibitory activities of 18 fractions of HILIC_JSPHs_90%ACN+0.1%FA separated using reversed phase-high performance liquid chromatography, where different lowercase letters above columns indicate significant ($p < 0.05$) differences between treatments and error bars = ± SD.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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